VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION:

Paragraph beginning at line 7 of page 7 has been amended as follows:

In a preferred embodiment, cells playing an important role in the induction of the immune response, such as APC, dendritic cells etc., are genetically engineered with vectors encoding the peptides of the invention (for example viral or retroviral vectors, such as those from adenovirus or lentivirus or MLV). Furthermore, the peptides can also be fused with a suitable protein carrier, to have a satisfactory processing and expression at the cell surface. Accordingly, the DNA encoding for the epitopes of the invention, may be inserted in a suitable expression vector, under the control of a suitable viral promoter, such as CMV or SV40, when a very efficient expression is required, or an inducible promoter such as that controlled by ecdysone. The epitopes herein referred correspond to the nucleotide fragments listed in the following Table 1 <u>(SEQ ID NOS 1-11, respectively, as they appear)</u>, according to the human MAGE-3 gene sequence deposited at GenBank under the accession number U03735:.

Heading appearing at line 1 of page 12 has been amended as follows:

Table 2: Determination of HLA-DR binding by MAGE-3 derived peptides (SEQ ID NOS 3-5, 7, 10, 1-2, 6, 8-9, 11, respectively, as they appear).

IN THE CLAIMS:

Claim 1 has been amended as follows:

1. (amended) Peptides binding MHC class II molecules selected from the group consisting of (SEQ ID NOS 1-11, respectively, as they appear):

a) EALGLVGAQAPATEE

b) RKVAELVHFLLLKYR

c) GNWQYFFPVIFSKAS

d) FFPVIFSKASSSLQL

e) SSLQLVFGIELMEVD

f) VGFIELMEVDPIGHL

g) PIGHLYIFATCLGLS

h) GDNQIMPKAGLLIIV

i) VQENYLEYRQVPGSD

j) TSYVKVLHHMVKISG

k) VLHHMVKISGGPHIS

MAGE-3 DERIVED IMMUNOGENIC PEPTIDES PRESENTED BY MHC OF CLASS II AND THE USE THEREOF

The present invention relates to peptides derived from MAGE-3 protein and to the use thereof as immunostimulants, specifically as agents capable of stimulating the CD4$^+$ T cell immune response.

The importance of CD4$^+$ T lymphocytes in anti-tumor immunity has been clearly demonstrated in animal models. CD4$^+$ T cells exert helper activity for the induction and maintenance of anti-tumor CD8$^+$ T cells (Greenberg, P.D., 1991,. Adv. Immunol. 49:281-355; Chen, P., et al., 1993, J. Immunol. 151:244-255; Mandelboim, O., et al., 1995,. Nat. Med. 1:1179-1183; Mayordomo, J.I., et al., 1995, Nat. Med. 1:1297-1302; Bellone, M., et al., 1997,. J. Immunol. 158:783-789; Ostrand-Rosemberg, S., et al., 1990, J. Immunol. 144:4068-4071; James, R., et al., 1991, Immunology 72:213-218), but they may also have an effector function either by indirect mechanism against MHC class II negative tumors, via macrophages activation, or by direct mechanism against MHC class II positive tumors.

Recently, the requirement of cognate CD4$^+$ T cell help for optimal induction of anti-tumor CD8$^+$ CTL was demonstrated (Ossendorp, F., et al. 1998. J. Exp. Med. 187:693-702). In humans, evidence for a role of CD4$^+$ T cells in anti-tumor immunity comes from the study of tumor infiltrating lymphocytes, which revealed the presence of both CD8$^+$ and CD4$^+$ T cells at the tumor site (Goedegebuure, P.S., et al. 1995. Immunol. Res. 14:119-131; Maccalli, C., et al. 1994. Int. J. Cancer 57:56-62), and from the detection in the sera of neoplastic patients of antibodies directed against tumor antigens (Sahin U. et al., 1997, Curr. Opin. Immunol. 9:709-716). However, in recent years research on the T cell immunity against human tumors has

focused mainly on identification of $CD8^+$ HLA class I restricted CTL responses. For example, WO 95/19783 discloses MAGE-3 derived peptides capable of binding to MHC class I molecules, such as the allele HLA-A1. Such peptides usually have a number of residues ranging from 8 to 10 amino acids.

To date tyrosinase, a tissue-specific antigen expressed in normal and neoplastic cells of melanocytic lineage (Topalian, S.L., et al. 1994. Proc. Natl. Acad. Sci. USA 91:9461-9465.; Yee, C., et al. 1996. J. Immunol. 157:4079-4086), is the only melanoma associated antigen demonstrated as a specific target for $CD4^+$ melanoma reactive T cells and for which $CD4^+$ T cell epitopes have been identified (Topalian, S.L., et al. 1996. J. Exp. Med. 183:1965-1971). WO 97/11669 (Topalian et al.) reports that peptides from this antigen are recognized in association with MHC class II molecules.

Characterization of the $CD4^+$ T cell epitope repertoire on other tumor associated antigens, expecially those that are tumor-specific and shared among tumors of several histotypes (Van den Eynde, B.J., et al. 1997. Immunol. Today 9:684-693), would contribute decisively to improve the efficacy of peptide-based immunization protocols in neoplastic patients.

The family of MAGE genes ("Melanoma Associated Antigen") consists of about 12 members which are expressed in various types of tumors. MAGE-3 is a tumor-specific antigen encoded by a gene expressed in a high proportion of melanomas and in several other tumor histotypes (head and neck squamous cell carcinomas, bladder carcinomas, lung carcinomas and sarcomas) and not in normal tissues, with the exception of testis and placenta (Van den Eynde, B.J., et al. 1997. Immunol. Today 9:684-693). $CD8^+$ CTL from melanoma patients recognize HLA class I restricted MAGE-3

epitopes (Van den Eynde, B.J., et al. 1997. Immunol. Today 9:684-693), and clinical trials with synthetic peptides corresponding to HLA-A1 and/or -A2 MAGE-3 binding sequences are ongoing in patients affected by melanoma and other neoplastic diseases (Van den Eynde, B.J., et al. 1997. Immunol. Today 9:684-693).

According to a first aspect, the invention relates to MAGE-3 derived immunogenic peptides capable of binding to MHC class II molecules. Such peptides have length from 12 to 15 residues and correspond to MAGE-3 fragments (according to the amino acid sequence reported in Gaugler B. et al. 1994, J. Exp. Med. 179, 921-930) 21-35, 111-125, 161-175, 251-265, 286-300, preferably 141-155, 146-160, 156-170, more preferably 171-185, 191-205 and 281-295. The corresponding amino acid sequences are reported in SEQ ID No. 1-11.

The peptides of the invention are characterized by promiscuous binding to different alleles of MHC class II molecules, such characteristic being advantageous in that one same peptide can be recognized by a wider patient population.

In an <u>in vitro</u> binding assay, the peptides of the invention proved capable of binding different purified molecules belonging to widespread HLA-DR alleles, and of inducing activation of $CD4^+$ cells. More particularly, it has been observed that stimulation with the peptides of the invention induces a remarkable proliferation of $CD4^+$ T cells and of their cytolytic activity. CD4+ T cells exposed to such peptides were able to cause lysis of melanoma cells expressing the MAGE-3 protein and the HLA-DR molecules. Details of such experimental evidence are reported in the examples.

The peptides are preferably prepared synthetically, for example according to the procedures described in

Merrifield, (1986) Science 232:341-347, and Barany and Merrifield, The Peptides, Gross and Meienhofer, eds (N.Y., Academic Press), pp. 1-284 (1979). The synthesis can be carried out in solution or in solid phase or with an automatized synthesizer (Stewart and Young, Solid Phase Peptide Synthesis, 2nd ed., Rockford Ill., Pierce Chemical Co. (1984). Alternatively, the recombinant DNA technology can be used, or the peptides can be prepared starting from the natural protein by fragmentation or enzymatic digestion. Furthermore, the amino acid residues can be replaced, preferably conservatively, by other residues of L- or D- amino acids, or added to the disclosed peptides, or they can be chemically modified, for example by amidation of the terminal carboxylic group or by binding with lipophilic groups (e.g. myristyl), or by glycosylation or conjugations with other peptides, to obtain more favourable properties, such as higher affinity to the MHC molecule, higher immunogenicity, better selectivity in inducing the immune response or higher bioavailability after administration. The peptides of the invention can also be chemically derivatized at the side chains which are therefore modified. For example, free carboxylic groups can be derivatized to form salts, methyl- and ethyl- esters or other types of esters or hydrazides.

The peptides of the invention can also be conjugated with known epitopes, for example with epitopes binding HLA molecules of class I, in order to induce a more complete spectrum of responses, of both the cytotoxic and helper type, and to enhance the response against the tumour.

The provision of new epitopes from an antigen not significantly expressed in normal tissues, such as MAGE-3, would allow to prepare vaccines for use in immunotherapy of patients with tumors expressing the same antigen. Furthermore, the $CD4^+$ T cells response induced by the

epitopes is strenghtened in that those cells, in addition to an intrinsic cytotoxic activity, exhibit also helper activity through the stimulation and proliferation of other T cells, such as $CD8^{+}T$ cells, as well as through macrophages activation.

Therefore, according to a further aspect, the invention provides pharmaceutical compositions containing an effective amount of a peptide of the invention, optionally in combination with other known peptides binding MHC class I molecules and corresponding to $CD8^{+}$ T cell epitopes, such as the peptides described in WO95/19783. In addition to the active ingredients , the compositions will contain pharmaceutically acceptable excipients. "Effective amount" herein means a sufficient amount to activate specific lymphocytes and induce an effective response against the tumor. Such an amount will depend on the peptide used, the administration, the severity of the disease to be treated and the general conditions of the patient and will usually range from 1 to 50 μg/ml, for example in case of peptides being loaded on dendritic cells.

According to a preferred embodiment, such compositions will be used for the preventive vaccination of patients with predisposition to neoplasias or in the therapeutical vaccination of neoplastic patients. "Vaccination" herein means both active immunization, i.e. the <u>in vivo</u> administration of the peptides to elicit an <u>in vivo</u> immune response directly in the patient, as in conventional vaccination protocols, for example against pathogens, and passive immunization, i.e. the use of the peptides to activate <u>in vitro</u> anti-tumor $CD4^{+}$ cells, which are subsequently re-inoculated into the patient.

The techniques for the preparation and the use of vaccines are known to those skilled in the art and are

described, per example, in Paul, Fundamental Immunology, Raven Press, New York (1989) or Cryz, S. J., Immunotherapy and Vaccines, VCH Verlagsgesselschaft (1991). Vaccines are conventionally prepared in the form of injectables, suspensions or solutions, but they can also be used in the form of solid preparations or liposomes. The immunogenic ingredients can be mixed with pharmacologically acceptable excipients, such as emulsifiers, buffering agents and adjuvants which increase the efficacy of the vaccine. The latter can be administered according to single or multiple dosage schedule. Multiple dose provides 1 to 10 separate doses, each containing a quantity of antigen varying from 1 μg to 1000 μg, followed by further doses at subsequent time intervals, necessary to maintain or to reinforce the immune response and, if required by the subject, a further dose after several months. In any case, the treatment regimen will depend on the response elicited in the treated patient, general conditions and progress of the tumor.

In a further aspect, the invention provides a method for inducing an immune response against tumor cells expressing a MAGE-3 antigen which comprises incubating APC cells (Antigen Presenting Cells) with the peptides of the invention in conditions suitable for the activation of effectors T CD4$^+$.

Such conditions comprise loading autologous APC with the peptides and the subsequent exposure to purified T CD4$^+$lymphocites . Suitable APC cells are autologous peripheral blood mononuclear cells (PBMC), dendritic cells, macrophages or activated B cells. The peptides are added to an APC culture for a sufficient time to obtain the peptide/APC binding, and subsequently a cell population containing CD4$^+$ CTLs is added, thereby causing activation and proliferation of CTLs. According to a preferred embodiment, T cells are taken from the treated patient and

optionally purified, then, after activation as described above and expansion in suitable culture medium, they are reintroduced in the same patient. Culture media can contain one or more cytokines (such as IL-2 or T-cell Growth Factor) which contribute to the expansion of $CD4^+$ precursors.

In a preferred embodiment, cells playing an important role in the induction of the immune response, such as APC, dendritic cells etc., are genetically engineered with vectors encoding the peptides of the invention (for example viral or retroviral vectors, such as those from adenovirus or lentivirus or MLV). Furthermore, the peptides can also be fused with a suitable protein carrier, to have a satisfactory processing and expression at the cell surface. Accordingly, the DNA encoding for the epitopes of the invention, may be inserted in a suitable expression vector, under the control of a suitable viral promoter, such as CMV or SV40, when a very efficient expression is required, or an inducible promoter such as that controlled by ecdysone.. The epitopes herein referred correspond to the nucleotide fragments listed in the following Table 1, according to the (human) MAGE-3 gene sequence deposited at GenBank under the accession number U03735:

INS B

TABLE 1

aa	Amino acid sequence	Nt
21-35	EALGLVGAQAPATEE	2525-2569
111-125	RKVAELVHFLLLKYR	2795-2839
141-155	GNWQYFFPVIFSKAS	2885-2929
146-160	FFPVIFSKASSSLQL	2900-2944
156-170	SSLQLVFGIELMEVD	2930-2974
161-175	VFGIELMEVDPIGHL	2945-2989
171-185	PIGHLYIFATCLGLS	2975-3019
191-205	GDNQIMPKAGLLIIV	3035-3079
251-265	VQENYLEYRQPVGSD	3215-3259
281-295	TSYVKVLHHMVKISG	3305-3349
286-300	VLHHMVKISGGPHIS	3320-3364

The invention also relates to antibodies, fragments or derivatives thereof, directed to the above described peptides. The general methodology for producing antibodies is well known and is disclosed per example in Kohler and Milstein, 1975, Nature 256, 494 or in J.G.R. Hurrel, Monoclonal Hybridoma Antibodies: Techniques and Applications, CRC Press Inc., Boco Raron, FL (1982). The antibodies can be polyclonal or, preferably, monoclonal, or antibody fragments like be F(ab')2, Fab, Fv or scFv.

Still a further aspect of the invention is a method for monitoring the frequency and the expansion of specific precursors for the peptides or the complete MAGE-3 protein, in neoplastic patients subject to active vaccination, by the ELISPOT technique (Herr, W. Et al., 1997, J. Immunol. Methods 203:141-52) or by cytofluorimetric analysis using tetramers consisting of tetrameric soluble molecules avidin-biotin-MHC class II, pulsed with the relevant peptide (Yee, C. et al. 1999, J. Immunol. 162:2227-2234).

Description of the Figures

Fig. 1: Proliferative activity of $CD4^+$ T cells challenged with the MAGE-3 Pool, tested in 2-d microproliferation assays.

The data are representative of (n=x) experiments, and are means of triplicate determinations ± SD. Panel A (n=6): responses to MAGE-3 Pool (0.01, 0.5, 0.1, 0.5, 1 and 5 μg/ml). Panel B (n=3): responses to recombinant MAGE-3 protein (5, 10 and 20 μg/ml). Panel C (n=7): responses to the individual synthetic peptides forming the MAGE-3 Pool (10 μg/ml) at different weeks of propagation. The blank (i.e. the basal level of proliferation of $CD4^+$ T cells in the presence of APC only) was subtracted and was as follows: 2 weeks: 30,866±1,115; 4 weeks: 7,106±2,201; and 6 weeks: 21,838±2,767. Asterisks indicate responses significantly higher than the blanks (*, P<0.001 and ** P<0.025, as determined by unpaired, one-tailed Student's t test). Panel D (n=5): response to MAGE-3 Pool (5 μg/ml) (a) and to peptide corresponding to sequence 281-295 (b), in the presence of different doses of L243 mAb (0.25 and 0.5 μg/ml). The blank was 1,251±444, the proliferation of $CD4^+$ T cells in the presence of MAGE-3 pool was 28,191±373, and the proliferation in the presence of sequence 281-295 was 22,504±141.

Fig. 2: Cytolytic activity of MAGE-3 specific $CD4^+$ T cells.

The data are representative of (n=x) experiments, and are means of triplicate determinations±SD. Panel A (n=6): lytic activity against different HLA-DR matched and unmatched melanoma cells. HLA-DR types of $CD4^+$ T cells and melanomas are indicated at the bottom along with their symbols.

Fig. 3: $CD4^{\pm}$ T cells recognize MAGE-3 (281-295) in association with HLA-DR11 on OI TC cells.

The data are representative of (n=x) experiments, and

are means of triplicate determinations±SD. Panel A (n=3): lytic activity of $CD4^+$ CTL against LCL alone or LCL pulsed with MAGE-$3_{141-154}$, MAGE-$3_{146-160}$ and MAGE-$3_{281-295}$. Panel B (n=3): cold target inhibition experiments. Cold targets [OI TC (circles) and LCL pulsed with MAGE-$3_{281-295}$ (squares)] were used to inhibit the lytic activity of MAGE-3 specific $CD4^+$ CTL against hot OI TC (E/T ratio was 40:1). Percentage of specific lysis against OI TC cells in the absence of cold targets was 26±1.2%.

For the abbreviations of HLA phenotypes and of cell lines see Example 4.

The following examples illustrate the invention in greater detail.

EXAMPLES

Example 1

DR-Peptide binding assay.

Peptide interactions with detergent-solubilized DR molecules were measured using an ELISA-based high-flux competition assay (Radrizzani, L., et al. 1997. J. Immunol. 159:703-711). HLA-DR molecules were isolated from the following human lymphoblastoid cell lines (LCL): DR1 (DRB1*0101) from HOM-2, DR3 (DRB1*0301) from WT49, DR4 (DRB1*0401) from PREISS, DR5 (DRB1*1101) from SWEIG, DR7 (DRB1*0701) from EKR, and DR8 (DRB1*0801) from BM9. DR2 (DRB1*1501) was isolated from the L cell transfectant L466.1. The molecules were affinity purified using the mAb 1-1C4 (Cammarota, G., et al. 1992. Nature 356:799-801), as described in (Sinigaglia, F., et al. 1992. Methods Enzymol. 203:370-386). Peptide competition assay was conducted to measure the ability of unlabeled peptides to compete with a biotinylated indicator peptide for binding to purified DR molecules. The following biotinylated indicator peptides were used: $GFKA_7$ for DR1 and DR7; $GIRA_2YA_4$ for DR2; $LAYDA_5$ for DR3; UD4 for DR4 (Hammer, J., et al. 1995. J. Exp. Med.

181:1847-1855); TT 830-843 for DR5; and $GYRA_6L$ for DR8. The biotinylated indicator peptide and HLA-DR molecules were incubated with 10-fold dilutions (0.001-100 mM) of the unlabeled competitor peptides (peptides corresponding to the MAGE-3 predicted sequences). To determine peptide binding affinity, the promiscuous $HA_{307-319}$ peptide from influenza hemagglutinin (Roche, P.A., et al. 1990. J. Immunol. 144:1849-1856) was included in each competition assay. The relative binding data of the unlabeled competitor peptides were expressed as inhibitory concentration (IC_{50}): i.e. the concentration of competitor peptide required to inhibit 50% of binding of the biotinylated indicator peptide.

The results of the binding assay are reported in the following Table 2.

INS
B2

~~Table 2: Determination of HLA-DR binding by MAGE-3 derived peptides~~

~~HLA-DR alleles~~

Residues	Sequence	*0101	*0301	*0401	*0701	*0801	*1101	*1501
141-155	GNWQYFFPVIFSKAS	25	>100(A)	7	0.1	3.2	0.6	3
146-160	FFPVIFSKASSSLQL	10	7	2	0.01	1.5	1.8	0.2
156-170	SSLQLVFGIELMEVD	7	90	45	0.03	7	28	0.18
171-185	PIGHLYIFATCLGLS	0.3	2.8	0.9	0.01	1.5	0.9	0.03
281-295	TSYVKVLHHMVKISG	15	26	70	0.02	0.01	0.03	0.5
21-35	EALGLVGAQAPATEE	14	>100	>100	25	>100	>100	22
111-125	RKVAELVHFLLLKYR	>100	>100	>100	55	7	0.7	0.055
161-175	VFGIELMEVDPIGHL	>100	0.6	28	10	100	>100	100
191-205	GDNQIMPKAGLLIIV	>100	>100	>100	6	1	4	0.07
251-265	VQENYLEYRQVPGSD	>100	>100	>100	26	10	60	5
286-300	VLHHMVKISGGPHIS	15	>100	>100	0.01	14	0.2	0.48

The binding data are expressed as relative binding capability (IC_{50} μM), calculated as concentration of competitor peptide required to inhibit 50% of binding of the biotinylated indicator peptide (indicator peptide). (a) IC_{50} values higher than 100 μM are outside the sensitivity limits of the binding assay.

Example 2

Peptide synthesis.

Peptides were synthetized on a 9050 Millipore synthesizer (Millipore Volketswil, Switzerland). The purity of the peptides was evaluated by RP-HPLC and electron spray mass spectrometry. Synthetic peptides were lyophilized and then reconstituted in DMSO at 2 mg/ml concentration and diluted in PBS as needed.

Example 3

Propagation of $CD4^{\pm}$ T cells and proliferation assay.

The synthetic peptides corresponding to the MAGE-3 sequences most promiscuous (141, 155, 146-160, 156-170, 171-185, 281-295) for HLA-DR binding (see Tables 1 and 2) were pooled (MAGE-3 Pool) and used to stimulate the PBMC of an healthy donor whose HLA type, identified by standard serologic typing, is: A1, A2/B41, B52/DR11, as described in Protti, M.P., et al. 1990. J. Immunol. 144:1711-1720. Briefly, $20x10^6$ PBMC were cultivated for 7 days in RPMI 1460 (GIBCO, Grand Island, NY) supplemented with 10% heat inactivated human serum (Technogenetics, Milan, Italy), 2mM l-glutamine, 100 U/ml penicillin, 50 μg/ml streptomycin (Biowhittaker, Walkersville, MD) (TCM) containing the MAGE-3 Pool (1 μg/ml of each peptide). The reactive lymphoblasts were isolated on a Percoll gradient (Protti, M.P., et al. 1990. J. Immunol. 144:1711-1720), further expanded in T cell growth factor (Lymphocult, Biotest Diagnostic Inc., Dreieich, West Germany) and restimulated at weekly intervals with the same amount of antigen plus irradiated (4000 rad) autologous PBMC as APC.

In the proliferation assay $CD4^+$ T cells and autologous irradiated PBMC were diluted in TCM to

2×10^5/ml and 2×10^6/ml, respectively and plated in triplicate in 96 round-bottom well plates (100 μl of $CD4^+$ T cells and 100 μl of APC). The cells were stimulated with different concentrations of MAGE-3 pool (0.05, 0.1, 0.5, 1 and 5 μg/ml), each peptide (10 μg/ml) and different concentrations of rMAGE-3 protein (5, 10 and 20 μg/ml). Triplicate wells with $CD4^+$ T cells alone and APC alone were used as controls. Three wells with $CD4^+$ T cells plus APC did not receive any stimulus to determine the basal growth rate (blank). In inhibition experiments different concentrations of mAb L243 or an isotype matched irrelevant mAb (0.25 and 0.5 μg/ml) were added in triplicate wells of $CD4^+$ cells plus APC stimulated with MAGE-3 pool (5 μg/ml) or MAGE-$3_{281-295}$ (10 μg/ml). After three days the cultures were pulsed for 16 h with $[^3H]$TdR (1 mCi, well, 6.7 Ci/mol, Amersham Corp., Milan, Italy). The cells were collected with a Skatron Titertek multiple harvester (Skatron Inc., Sterling, VA) and the thymidine incorporated was measured in a liquid scintillation counter.

T cells were 94% $CD4^+$ after 1 week of culture and could be propagated in long term culture by weekly restimulation with the MAGE-3 Pool in the presence of autologous irradiated PBMC. In microproliferation assays (Fig. 1) the cells responded vigorously to the MAGE-3 Pool (Panel A), even at low concentrations (100-500 ng/ml). Reactivity to the individual peptides forming the pool was also periodically investigated (Panel C): the $CD4^+$ T cells predominantly recognized the peptide corresponding to MAGE-$3_{281-295}$ and, although to a much lower extent, the peptides corresponding to the overlapping sequences MAGE-$3_{141-154}$ and MAGE-$3_{146-160}$.

Reactivity to MAGE-$3_{281-295}$ increased during the propagation of the line (Panel C). The proliferative activity of $CD4^+$ T cells in the presence of MAGE-3 Pool (Panel Da) or MAGE-$3_{281-295}$ (Panel Db) was inhibited by addition in culture of different concentrations of L243 mAb (Panel D), demonstrating that the recognition of MAGE-3 sequences was HLA-DR restricted.

The HLA-DR11+ PBMC from the healthy donor were also stimulated with a second pool of synthetic peptides corresponding to the MAGE-3 sequences 21-35, 111-125, 161-175, 191-205, 251-265 and 286-300. The $CD4^+$ T cells proliferated in a dose dependent manner to different concentrations of the MAGE-3 pool II, and the study of the epitope repertoire of the MAGE-3 specific $CD4^+$ T cells showed recognition of sequences MAGE-$3_{111-125}$, MAGE-$3_{161-175}$ and predominantly MAGE-$3_{191-205}$. Furthermore, MAGE-3 specific $CD4^+$ T cells from a melanoma patient, whose HLA-DR type is HLA-DR4/DR11, recognized the sequences MAGE-$3_{141-155}$, MAGE-$3_{146-160}$, MAGE-$3_{156-170}$, MAGE-$3_{171-185}$ and MAGE-$3_{281-295}$. The study of the restriction element showed that all sequences were recognized in association with the HLA-DR4 allele, demonstrating that sequences 141-155, 146-160 and 281-295 are presented to $CD4^+$ T cells in association at least with two different alleles (HLA-DR11 and HLA-DR 4).

Example 4

Cytotoxicity assay

$CD4^+$ T cells were tested for specific lytic activity in a standard 4-h ^{51}Cr release assay as described in Protti, M.P., et al. 1996. Cancer Res. 56:1210-1213. The following targets were used: melanoma

cells (SK-Mel 28, HT144, OI TC described in Imro, M.A., et al. 1998. Hum. Gene Ther. 9:1335-1344 and MD TC established in our laboratory from a cutaneous metastasis), and LCL. The HLA-DR type of target cells, identified by molecular or serologic typing, was: SK-Mel 28 (DR*04*13), HT144 (DR*04*07), OI TC (DR*01*11), MD TC (DR*04*11), LCL (DR11). In cold target competition assays, unlabeled target cells (cold targets) were seeded in plates at serial ratios of hot-to-cold target cells. Effector $CD4^+$ T cells and ^{51}Cr-labeled target cells (hot targets) were then added, and cytotoxicity assessed as described above. Percentage inhibition was calculated as follows:

[(% specific lysis without cold target-% specific lysis with cold target)/(% specific lysis without cold target)]x100.

$CD4^+$ T cells showed cytolytic activity against OI TC and MD TC which express the HLA-DR11 restricting allele, while they did not kill SK-Mel 28 and HT144 which express unrelated HLA-DR alleles (Figure 2a). To verify whether the cytolytic $CD4^+$ T cells recognized HLA-DR11 restricted MAGE-3 epitopes on melanoma cells, first was tested their lytic activity against HLA-$DR11^+$ LCL unpulsed, or pulsed with the synthetic peptides recognized in microproliferation assays. LCL pulsed with MAGE-$3_{281-295}$ were strongly recognized by the $CD4^+$ T cells, while no killing activity against LCL unpulsed or pulsed with MAGE-$3_{141-154}$ and MAGE-$3_{146-160}$ was detectable (Figure 3a). Subsequently, cold target inhibition experiments were performed which showed that the lytic activity of $CD4^+$ T cells against OI TC was inhibited by the addition of LCL pulsed with MAGE-3_{281-}

295 (Figure 3b), demonstrating that this sequence is indeed presented by HLA-DR11 on the OI TC melanoma cells. These results further demonstrate that MAGE-$3_{281-295}$ is naturally processed and forms a cytotoxic $CD4^+$ T cell epitope.

$CD4^+$ T cells specific for sequence MAGE-$3_{191-205}$ also showed cytolytic activity against MAGE-3/HLA-DR11+ melanoma cells and cold/target inhibition experiments showed that the sequence 191-205 was indeed recognized at the surface of the melanoma cells in association with the HLA-DR11 allele and therefore this epitope is naturally processed.

In the case of the patient, the $CD4^+$ T cells showed cytolytic activity against the autologous tumor that expresses the MAGE-3 antigen, and against the SK-Mel 28 melanoma cells that express the antigen and the HLA-DR4 retriction allele, while they did not kill melanoma cells expressing the MAGE-3 protein but an unrelated HLA-DR allele.